Biochimica et Biophysica Acta, 293 (1973) 424–433

BBA 66834

ARYLESTERASE AND ACETYLCHOLINESTERASE IN THE ERYTHROCYTES OF MAN, COW AND PIG

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(Received July 27th, 1972)

SUMMARY

1. The esterase activities of human, bovine and porcine erythrocytes have been determined gasometrically with phenyl acetate and acetylcholine iodide as substrates. The activity of human erythrocytes is twice that of bovine and porcine cells. Intact cells and hemolysates have about the same activity. The three species differ in regard to the readiness of enzyme release from the cells by simple hemolysis against a hypotonic solution.

2. The effect of ultrasonic waves, together with other results obtained, suggest that both arylesterase (aryl-ester hydrolase, EC 3.1.1.2) and acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) are probably bound to the erythrocyte membrane.

3. The inhibitory effects of physostigmine and mercuric ions suggest that 10–20% of the esterase activity, measured with phenyl acetate, is due to arylesterase (or carboxylesterase). The three species studied differ slightly regarding the relative activity level of each esterase type.

4. Hemoglobin-free esterase fractions have been prepared by gel filtration on Sephadex G-100. The properties and composition of these fractions have been studied with thiophenyl acetate and acetylthiocholine iodide as substrates using a quantitative spectrophotometric technique as well as a histochemical one for disc electropherograms.

INTRODUCTION

The erythrocytes of man and other mammalian species contain, in addition to a membrane bound acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7), other relatively non-specific and less well-characterized esterases. The existence of an aromatic esterase (aryl-ester hydrolase, EC 3.1.1.2) in human erythrocytes has been known for many years[1], and this enzyme is probably not identical with the arylesterase present in human plasma[2–4]. The acetylcholinesterase is strongly bound to

the cell membrane[5–7], but the localization of the arylesterase in the red cell has not been investigated.

As a first step in an attempt to compare the localization and properties of arylesterases and acetylcholinesterases in the erythrocytes of various species, experiments have been carried out with human, bovine and porcine red cells. Our attempts to remove these enzymes from the cells (by various methods) revealed great difference in properties of erythrocytes from species to species. Since the appearance of our first observation along these lines using human and bovine erythrocytes, a study on acetylcholinesterase has been published[8] that suggests that a fundamental difference in the structure of the two erythrocyte membranes is responsible for the much easier release of this enzyme from the bovine cell membrane as compared with the difficulty in releasing acetylcholinesterase from the human membrane. More recently, the characteristics of this enzyme in erythrocytes of the three species included in the present investigation were discussed[9]. We have confirmed these previous results in a somewhat different study with acetylcholinesterase and have demonstrated that arylesterase probably also is a membrane-bound enzyme which follows closely the first enzyme in an unexpected way.

MATERIALS AND METHODS

Erythrocyte suspension

Heparinised freshly-drawn blood was centrifuged at 1500 × *g* for 10 min, and the plasma and buffy coat were removed. The red cells were washed three times with 0.90% NaCl and resuspended in this solution to give the original hematocrit value.

Substrates and esterase inhibitors

Acetylcholine iodide and acetylthiocholine iodide were obtained from Sigma Chemical Co. Acetylcholine was used as a 4.0 mM solution and the thiol ester as a 22.5 mM solution. Phenyl acetate and thiophenyl acetate were synthesised according to a recent report[10] or were purchased from Fluka (phenyl acetate) and Polysciences Inc. (thiophenyl acetate), respectively. Phenyl acetate was used as a 12.5 mM suspension and thiophenyl acetate as a 0.15 M methanolic solution. Physostigmine salicylate and mercuric chloride were commercial products.

Other reagents

Sephadex G-100 was purchased from Pharmacia (Uppsala), and acrylamide, bisacrylamide and *N,N,N′,N′*-tetramethylethylene diamine were obtained from Fluka. All other chemicals employed were standard commercial products, and all solutions were prepared in double deionised water.

Assay of esterase activities

The esterase activity was measured by a manometric technique where the test solutions were highly coloured, and in all other cases by a spectrophotometric technique using thiol esters as substrates. Activity was expressed in nmoles of substrate hydrolysed per min per ml. According to the former method[11,12], the activity was measured at 25 °C in a sodium bicarbonate–CO_2 buffer of pH 7.4 with acetylcholine

iodide or phenyl acetate as substrates in a final concentration of 3.2 mM and 10 mM respectively. Corrections were made for spontaneous substrate hydrolysis.

The spectrophotometric assay of esterase activity was based on a technique described by Ellman *et al.*[13] for cholinesterases and recently applied in the quantitative assay of arylesterase activity[10]. The reaction mixture was composed of 3.0 ml of 0.10 M phosphate buffer, pH 8.0, containing 0.25 mM DTNB (3-carboxy-4-nitrophenyl disulfide; obtained from Sigma), 0.05 ml of the enzyme solution and 0.02 ml of the substrate solution (thiophenyl acetate or acetylthiocholine iodide), mixed in that order. The increase in absorbance at 412 nm was recorded with a Coleman spectrophotometer using a Servogor recorder (chart speed 3 cm/min), or, in some experiments, a Beckman DB-G spectrophotometer with a Contron 3012 recorder.

Determination of protein

The protein content in the fractions was determined according to the method of Lowry *et al.*[14] using bovine serum albumin as a standard.

Preparation of hemolysate and stroma

Washed erythrocytes were hemolysed in 5 times the volume of distilled water for 5 days. The hemolysate was then centrifuged at 100 000 $\times$ *g* for 30 min and the stroma washed with water, recentrifuged and suspended in water to the volume of the original hemolysate.

Sonication of erythrocyte hemolysate and stroma

For sonication, 3.0 ml of the hemolysate or a suspension of isolated stroma was pipetted into a sonication chamber. A current of 2.5 A was applied to the sonifier (Branson sonifier Model S125, Branson Instruments Inc., Stanford, Conn.), and the sonication time was 15 s and was repeated once. The temperature was kept at +2 °C.

Gel filtration

A column (2 cm $\times$ 70 cm) was packed with Sephadex G-100 (140 $\times$ 400 mesh) in the cold with the swollen gel and equilibrated with 0.04 M sodium acetate, pH 7.0, with a flow rate of about 20 ml per h, controlled by a peristaltic pump. The sample, 4.0 ml of stroma free hemolysate, was applied to the top of the column which had been previously covered with a filter-paper disk. Elution was carried with the same salt solution and at the same flow rate. Effluent was collected in 3 ml fractions. All columns were run at 0–5 °C.

Analytical polyacrylamide electrophoresis

The procedure used was a modification of a method described by Davis[15]. The electrophoresis was carried out at a current of 3 mA per gel in 6 mm $\times$ 80 mm glass tubes for 2.5 h. The gel was frozen about 30 min and then cut in two halves. One of these halves was stained for protein. The other half was stained for esterase activity by using a modification of a method described by Juul[16]. The gel was preincubated at room temperature for 30 min in phosphate buffer (70 mM, pH 6.1) containing 10 mM glycine, 2 mM $CuSO_4$, and 30 mM Na_2SO_3. Incubation was then continued for 90 min in the same solution containing 3.2 mM acetylthiocholine or thiophenyl acetate. Thereafter, the gel was immersed in 3 M ammonium sulphate at 4 °C for

24 h and then for the same period of time in the same sulphate solution saturated with dithiooxamide. Finally, the gel was treated for 24 h with 7% acetic acid.

RESULTS

Esterase activity of human, bovine and porcine erythrocytes

The esterase activity was determined in intact erythrocytes, in whole cell hemolysates, and in the stroma isolated from the hemolysates. The activity was

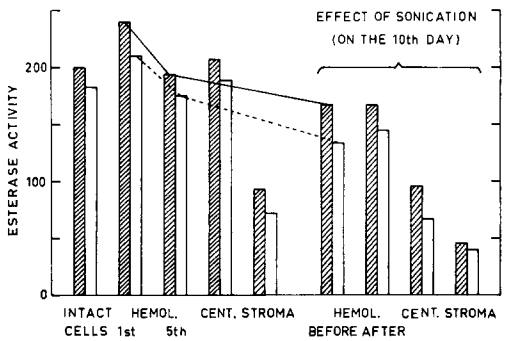


Fig. 1. Esterase activity of human erythrocytes, hemolysate and stroma measured with phenyl acetate (shaded areas) and acetylcholine iodide (open areas) as substrates using the gasometric technique. Esterase activity is expressed in nmoles of substrate split per min per ml erythrocytes based on original blood volume. Intact cells, hemolysate, and stroma were prepared according to the description in Materials and Methods. Sonication of the erythrocyte stroma was performed with a hemolysate kept in the cold (before sonication) for a period of time indicated on the graph. The centrifugate and stroma were prepared as described in the text. Activity values of hemolysates, kept at various length of time in the cold before used, are connected with a full (phenyl acetate) or a dotted line (acetylcholine).

measured with the gasometric technique using phenyl acetate and acetylcholine iodide as substrates. The results are presented in Figs 1–3. In comparing the activities for each preparation measured with the two substrates, the levels should be regarded as relative ones as the substrate concentrations used were arbitrarily chosen.

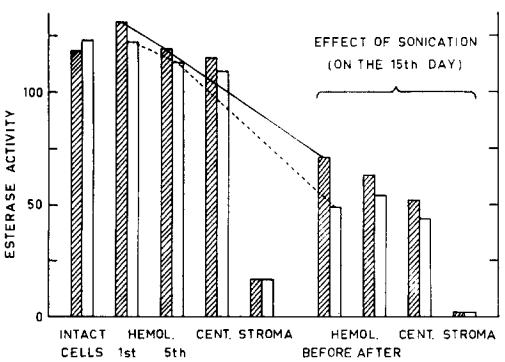


Fig. 2. Esterase activity of bovine erythrocytes, hemolysate and stroma. See Fig. 1 for further explanation. *N.B.* The activity scale is different from that in Fig. 1.

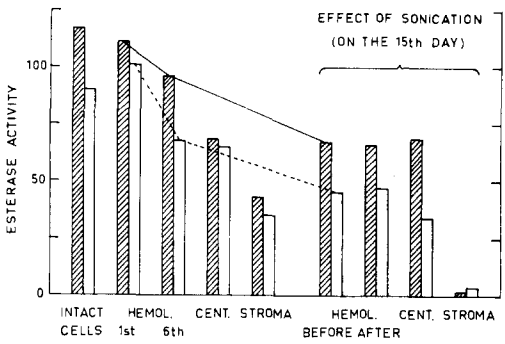


Fig. 3. Esterase activity of porcine erythrocytes, hemolysate and stroma. See Fig. 1 for further explanation. *N.B.* The activity scale is different from that in Fig. 1.

Intact cells and hemolysate had about the same activity though the activity was somewhat lower for intact cells in case of human and bovine erythrocytes. The esterase activity of human erythrocytes was twice that of bovine and porcine cells. After high speed centrifugation the isolated stroma contained a relative high percentage of the activity. Bovine stroma retained less activity than the other two types of stroma studied. It will be noted that the relative activities measured with the two substrates were almost the same in the stroma, the centrifugate and the original material. The apparent higher activity of the "stroma + centrifugate" (most pronounced with human erythrocytes) is obscure and will be further investigated.

Ultra sonic waves had a very little effect on the activity of the hemolysate of all three species, nor were the relative activities obtained with the two substrates very much altered by this treatment. The disruption of the cells by this treatment seemed to be greatest in the case of bovine and porcine erythrocytes. An appreciable amount of activity was still bound to human stroma after sonication of the hemolysate. These results suggest that arylesterases might be bound to the cells (possibly the membrane).

Esterase activity of bovine stroma compared with the activity of acetylcholinesterase

The activity of carefully washed bovine stroma was measured with the gaso-

TABLE I

ESTERASE ACTIVITY OF BOVINE STROMA AND PURIFIED ACETYLCHOLINESTERASE FROM BOVINE ERYTHROCYTES ("SIGMA") EXPRESSED IN nMOLES OF SUBSTRATE HYDROLYZED PER MIN PER ML STROMA SUSPENSION CALCULATED ON BLOOD VOLUME, OR MG PROTEIN, RESPECTIVELY. Ph, AROMATIC ESTER; Ch, CHOLINE ESTER

Substrate	*Stroma*		*Acetylcholinesterase*	
	Activity	*Ph/Ch*	*Activity*	*Ph/Ch*
Phenyl acetate	39.0		180	
		1.97		1.12
Acetylcholine iodide	19.8		160	
Thiophenyl acetate	36		140	
		0.4		0.25
Acetylthiocholine iodide	90		520	

metric and the spectrophotometric techniques and the results compared with those obtained in a similar manner with a purified acetylcholinesterase preparation from the same source. The results are presented in Table I. With both techniques the relative activities against aromatic esters were higher for the stroma than for the arylesterase-free preparation. This could be taken as indirect evidence that the stroma contained an esterase different from acetylcholinesterase and hydrolysing phenyl acetate and thiophenyl acetate.

Effect of inhibitors on the esterase activity of the hemolysate

The effects of physostigmine, a selective cholinesterase inhibitor, and mercuric ions, a relatively selective inhibitor of arylesterases[17], were measured using freshly prepared hemolysates of the three types of erythrocytes and phenyl acetate as substrate. The results, presented in Fig. 4, show that about 75% of the activity ob-

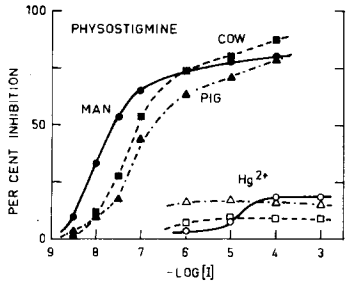


Fig. 4. Effect of physostigmine salicylate and $HgCl_2$ on the esterase activity of freshly prepared hemolysates measured gasometrically with phenyl acetate as substrate. All incubations of the hemolysate with the inhibitor (at the concentration indicated on the abscissa) were carried out at 30 °C for 50 min before the substrate was added and the esterase assay started. Filled symbols represent physostigmine as inhibitor; open symbols, $HgCl_2$ as inhibitor. ○—○, man; □—□, cow; △—△, pig.

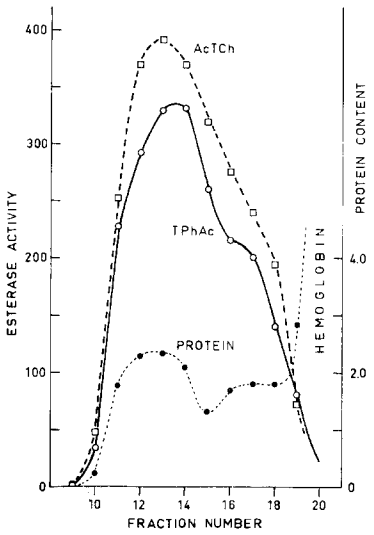
tained with this noncholine ester was due to acetylcholinesterase. There were only small differences in the inhibition curves with the two inhibitors for the three species. Porcine erythrocytes probably contained relatively more arylesterases and bovine erythrocytes more acetylcholinesterase than the two other species. The S-shaped curve obtained for the sensitivity of human erythrocyte esterases to Hg^{2+} suggests that two phenyl-acetate hydrolysing enzymes with different sensitivity to this ion were present.

Preparation of hemoglobin-free esterase fractions

Representative elution profiles obtained by gel filtration with hemolysates of the three species studied are illustrated in Figs 5, 7 and 9.

After 5 days at +4 °C, a 4 ml sample of a stroma-free hemolysate was applied to a column of Sephadex G-100. Elution was carried out with 0.04 M sodium acetate. It is significant that for bovine (Fig. 7) and porcine (Fig. 9) hemolysates hemoglobin was completely resolved from esterase proteins; in the case of human hemolysate this separation was not as complete.

It can be observed in Fig. 5 that the esterase fractions obtained with human hemolysate were actually more complex than the corresponding bovine and porcine



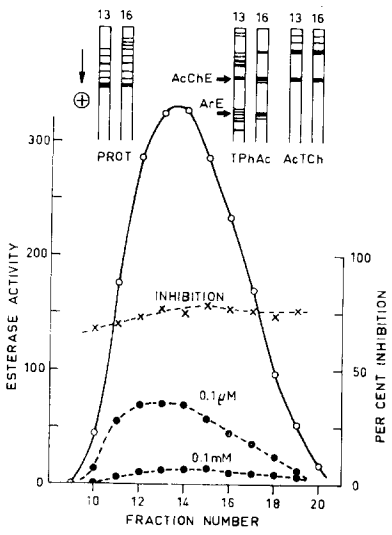


Fig. 5. Gel filtration of hemolysate of human erythrocytes on a column of Sephadex G-100. A 4 ml sample of the stroma-free hemolysate was applied to the column (2 cm × 70 cm). Elution was carried out with 0.04 M sodium acetate (pH 7.0) and the effluent was collected in 3 ml fractions (flow rate about 20 ml per h). Esterase activity expressed in nmoles of substrate hydrolysed per min per ml fraction, and protein content (●—●) in mg per ml. Substrates: thiophenyl acetate (TPhAc), ○—○; acetylthiocholine iodide (AcTCh), □—□.

Fig. 6. Effect of physostigmine salicylate on the esterase activity of fractions after gelfiltration of human hemolysate (Fig. 5). Activity measured with thiophenyl acetate as substrate. Control, (○—○); physostigmine present in concentrations as indicated, (●—●); percent inhibition curve with 0.1 μM physostigmine, (×—×). On top of the graph, polyacrylamide-gel electropherograms of two fractions as indicated. Protein (Prot), and esterase activity (thiophenyl acetate, TPhAc; acetylthiocholine iodide, AcTCh) developed as described in Materials and Methods. AcChE, acetylcholinesterase; ArE, arylesterase.

fractions. This is also obvious from the electropherograms in Figs 6, 8 and 10 which illustrate the presence of both acetylcholinesterases and arylesterases in all three species. Whether the existence of multiple bands of each esterase type is due to artifacts or to the presence of true isoenzymes cannot be evaluated at the present time.

As was reported above (*cf.* Fig. 4), most of the activity of the hemolysate measured with thiophenyl acetate or phenyl acetate was due to acetylcholinesterase. This was confirmed by the sensitivity to physostigmine of the activity of the fractions obtained by gel filtration, illustrated in Figs 6, 8 and 10. Fig. 6 shows that all active fractions were similarly sensitive to physostigmine in spite of the asymmetric esterase peak. This is an indication of the probable presence of at least two active acetylcholinesterase components of different molecular size. It was noted that a relatively better separation of cholinesterases and physostigmine-resistant components (probably arylesterases) was achieved for porcine (Fig. 10) than for human (Fig. 6) and bovine fractions (Fig. 8).

DISCUSSION

Among the various esterases identified in mammalian erythrocytes, acetyl-

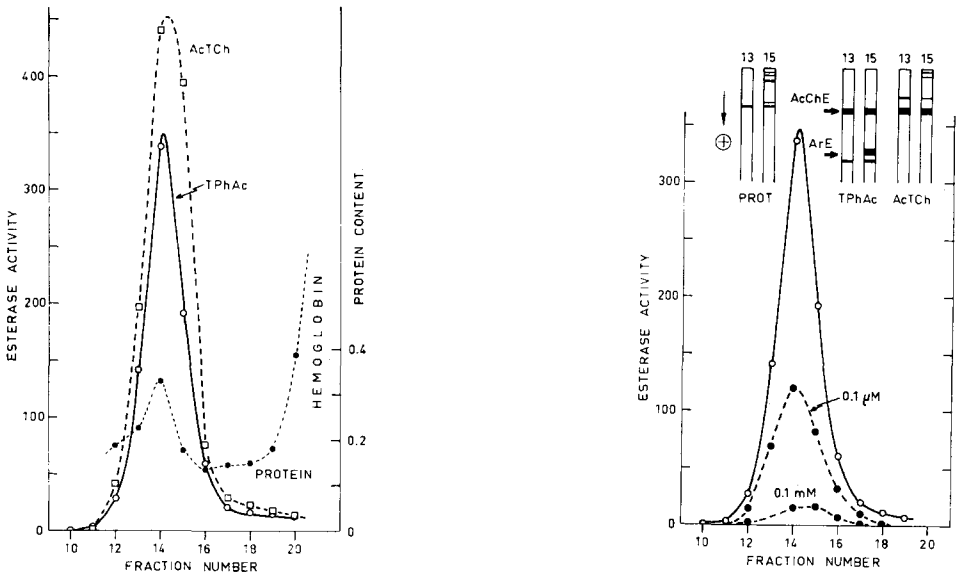


Fig. 7. Gel filtration of hemolysate of bovine erythrocytes. Experimental details and keys as indicated in Fig. 5.

Fig. 8. Effect of physostigmine salicylate on the esterase activity of fractions after gel filtration of bovine hemolysate (Fig. 7). Experimental details and keys as indicated in Fig. 6.

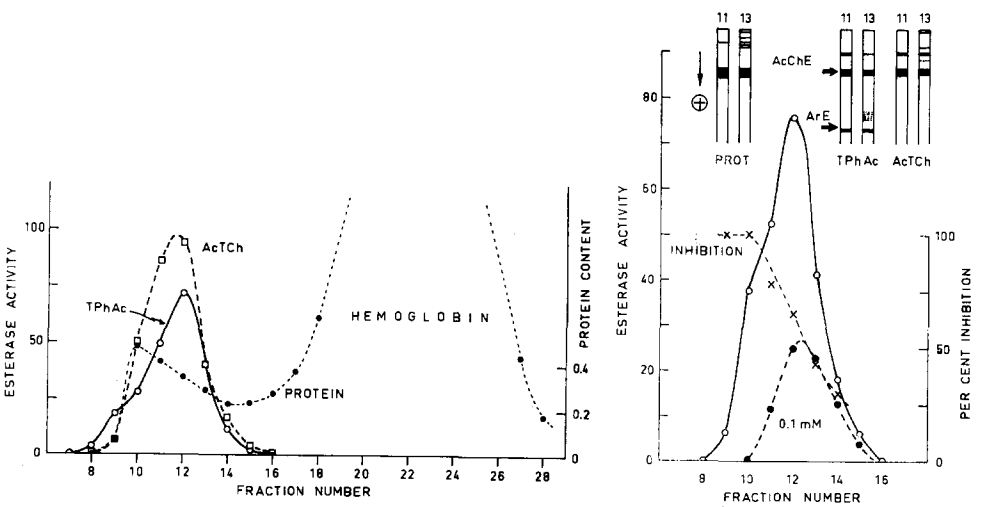


Fig. 9. Gel filtration of hemolysate of porcine erythrocytes. Experimental details and keys as indicated in Fig. 5.

Fig. 10. Effect of physostigmine salicylate on the esterase activity of fractions after gel filtration of porcine hemolysate (Fig. 9). Experimental details and keys as indicated in Fig. 6.

cholinesterase has been most thoroughly studied. There are a number of strong indications that this enzyme is associated with the membrane, an association which has rendered its purification difficult. Successful results have been published recently, however, reporting the solubilization of active acetylcholinesterase from human erythrocyte stroma[7,8]. Most interesting is the observation that membranes isolated from various species behave differently with regard to the readiness of the release of this enzyme and other membrane constituents[8,9].

We noticed this difference several years ago when working on the purification of arylesterase from human and bovine erythrocytes. Unless the results presented above are to be regarded as preliminary only they do not contradict that arylesterase might also be bound to the membrane. Moreover, the relative activity levels obtained with intact cells, hemolysate and stroma from the three species studied seem to indicate that bovine erythrocytes exhibit more intracellular arylesterase activity than the two other species. In addition, human erythrocytes seem to have these enzymes more tightly bound to the stroma than do bovine and porcine cells.

Arylesterase, in contrast to cholinesterases and carboxylesterases, are extremely sensitive to mercuric ions[17] and this test can be used to differentiate arylesterases from other esterase types when present in a mixture. The results obtained in the presence of mercuric ions indicate that 10–20% of the esterase activity measured with an aromatic non-choline ester is due to arylesterase (Fig. 4) and the relative amounts between this enzyme and acetylcholinesterase differ slightly for the three species studied.

Gel filtration is a most suitable technique for preparing esterase fractions free of hemoglobin and other low molecular size constituents of a hemolysate. Particularly good separation is obtained with bovine and porcine erythrocyte hemolysates. In the quantitative analysis of the fractions obtained we have found the thiol ester technique most useful[10]. These esters were also used to detect esterase active components on polyacrylamide electropherograms. The fractionation patterns (Figs 5–10) demonstrate that acetylcholinesterase and arylesterase follow each other rather closely. Both enzymes might exist as polymers and form aggregates as has been demonstrated for acetylcholinesterase[18,19]; this is under current investigation.

ACKNOWLEDGEMENT

This investigation was supported by grants (to K.-B.A.) from the Swedish Natural Science Research Council.

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